Lymphovascular Invasion in Colorectal Cancer: An Interobserver Variability Study

Published in final edited form as:
Am J Surg Pathol. 2008 December ; 32(12): 1816–1821. doi:10.1097/PAS.0b013e3181816083.

LYMPHOVASCULAR INVASION IN COLORECTAL CANCER: AN INTEROBSERVER VARIABILITY STUDY

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Abstract

Background—Lymphovascular invasion (LVI) in colorectal cancer (CRC) is considered a strong stage-independent prognostic factor and influences decisions regarding adjuvant chemotherapy in patients with Stage II tumors. However, the degree of interobserver agreement among pathologists for LVI in CRC is largely unknown. This study was undertaken to examine such interobserver variability, and we hypothesized that the use of immunohistochemical markers for vascular and lymphatic channels could improve interobserver agreement.

Design—Fifty cases of AJCC stage II moderately differentiated CRC from 1990 to 2005 from the pathology archives were selected; mucinous, medullary, and other recognized special subtypes were excluded. Fifty H&E slides (one from each case) were circulated to 6 GI pathologists, who independently assessed small and large vessel invasion. No diagnostic guidelines were given to the participating pathologists; each was instructed to apply the criteria for LVI that he or she used in daily practice. Immunohistochemistry (IHC) for D2-40 and CD31 was performed on corresponding paraffin blocks. The IHC slides were randomized, recirculated, and rescored for LVI. Results were analyzed by kappa (κ)statistics, which correct for agreement by chance, and for percent agreement.

Results—The average κ values were determined for the H&E slides (large and small vessel), CD31 (small vessel), and D2-40 (small vessel) (Figure 1). Agreement was fair for H&E small vessel invasion (κ = 0.28; 95%CI 0.22–0.34). The least agreement was seen in interpretation of H&E large vessel invasion (κ = 0.18; 95%CI 0.11–0.26). Agreement was not improved by use of immunohistochemical stains: CD31 (large vessel, κ = 0.42, 95%CI 0.20–0.63, small vessel, κ = 0.26, 95%CI 0.10–0.42) and D2-40 (κ = 0.32, 95%CI 0.21–0.42).

Conclusions—Interobserver variability in diagnosis of LVI was substantial on H&E slides and did not improve upon use of IHC. Agreement in evaluation of large vessel invasion was only slightly higher than would be seen by chance alone. This study highlights the need for criteria in evaluation of lymphovascular invasion, as this assessment may impact patient prognosis and thus change the course of clinical treatment.

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INTRODUCTION

The histological identification of lymphovascular invasion (LVI) by tumor has long been recognized as a potential prognostic indicator and predictor of patient outcome. Many studies have investigated the presence of lymphovascular invasion in colorectal cancer and have determined it to be a strong stage-independent prognostic marker (2,3,5,11–19,21). The presence of lymphatic invasion in malignant polyps has been associated with an increased risk of regional lymph node metastases (6,20,22), and tumor invasion of extramural veins has been recognized as a risk factor for liver metastases (24). A College of American Pathologists (CAP) Consensus Statement in 1999 reviewed the available literature and determined that "blood or lymphatic vessel invasion" warranted placement in Category I as a factor "definitively proven to be of prognostic import based on evidence from multiple statistically robust published trials and generally used in patient management."(5)

Given the importance of identifying LVI, it is crucial that pathologists agree on its identifying features. However, there are several caveats to the available literature on lymphovascular invasion. There is significant variability in recognition, diagnosis, and reporting of lymphovascular invasion, as well as in processing of specimens (1,3,4). The number of sections of tumor submitted in a resection specimen varies among institutions and may influence detection of LVI. The number of levels cut from each block of tissue also varies among practices. Pathologists may or may not choose to use ancillary stains such as elastin or immunohistochemical markers of endothelium in order to aid in assessment of LVI. There is also considerable interpretation variability among pathologists. The available published criteria for the histological diagnosis of LVI on examination of the hematoxylin&eosin (H&E)-stained slide include (5,24–28): presence of tumor cells within a vascular space; erythrocytes surrounding the tumor cells; identification of endothelial cells lining the space; the presence of an elastic lamina surrounding tumor; and attachment of tumor cells to the vascular wall. Although criteria for LVI are recognized, issues in interpretation include the determination of retraction artifact *versus* true vascular space; endothelial lining *versus* stromal fibroblasts; true vascular invasion *versus* "floater" (knife carryover artifact); and the presence or absence of a muscle wall. These difficulties in interpretation have been noted in the literature: a study of venous invasion in malignant colorectal polyps showed that there is no difference in patient survival and concluded that the criteria for diagnosis of vascular invasion is too "subjective" to be of clinical importance (8)

In addition to issues of interpretation, there are variations in reporting of LVI and in study methodology. Although the 1999 CAP Consensus Statement recommended reporting of both small and large vessel tumor invasion as well as location of LVI (5), these recommendations have not been universally adopted. Studies examining the link between LVI and patient outcome are also inconsistent. Many retrospective studies extract the reporting of LVI from the patient's medical record, without review by a central pathologist for consistency. Others make use of a single pathologist's interpretation of LVI without taking into account potential interobserver variability in diagnosis of LVI. And finally, most studies do not define the criteria used to determine the presence or absence of LVI. The variability in sampling, interpreting, diagnosing, reporting, and studying LVI leads to the obvious difficulty in determining its true prognostic value.

The variation in diagnosis of lymphovascular invasion affects clinical assessment of prognosis and may change the course of therapy for the patient (10,17,18,30). Interobserver studies investigating pathology reporting of lymphovascular invasion in breast cancer have been published (9,23). However, studies specifically addressing agreement in the diagnosis of LVI in colorectal cancer, using both H&E-stained slides and immunohistochemistry have not been undertaken previously. Our study aims to investigate interobserver agreement in the diagnosis

of LVI in colorectal carcinoma. The hypothesis is that the agreement among pathologists on examination of the H&E slides is poor, but the use of ancillary immunohistochemical stains CD31 and D2-40 increases both the diagnosis of LVI and the interobserver agreement, and ultimately improves patient care.

MATERIALS AND METHODS

This study was reviewed and approved by the Vanderbilt Institutional Review Board. Fifty cases of AJCC Stage II moderately-differentiated colorectal cancer diagnosed between the years 1990 and 2005 and identified from the pathology archives were randomly selected for study. Fifty cases were chosen in order to achieve reasonable statistical power, i.e., 80%, with an interobserver study involving six pathologists. Mucinous and medullary carcinoma subtypes were excluded in order to maintain some degree of uniformity in our sample population. Fifty de-identified H&E slides (one from each case, representing a full-thickness section of tumor) were circulated to six gastrointestinal pathologists in the United States. Each pathologist independently assessed small and large vessel invasion, using his or her own criteria; no diagnostic guidelines were provided.

Immunohistochemistry (IHC) for CD31 and D2-40 was then performed on the corresponding paraffin blocks. The slides were deparaffinized in xylene and rehydrated. Antigen retrieval was performed using a citrate buffer at pH 6.0 and pressure cooker for 15 minutes with a bench cool down of 10 minutes. A hydrogen peroxide quench using 0.03% H_2O_2 with sodium azide was performed for 5 minutes. A 20 minute incubation using serum-free Protein Block followed. A 30 minute incubation using the primary antibodies (Dako CD31 monoclonal mouse anti-human, CAT# M0823, at 1:200 dilution; Dako D2-40 monoclonal mouse anti-human, CAT# M3619, at 1:100 dilution) was performed. Secondary detection consisted of a 30 minute incubation using Dako Envision + HRP-labeled polymer and staining was visualized by 5 minute incubation with diaminobenzidine tetrahydrochloride (DAB). The slides were counterstained with Mayer's hematoxylin and coverslipped. Normal colon was used as a control slide for each immunohistochemical stain.

The IHC slides were then de-identified, randomized, recirculated, and rescored for the presence of small and large vessel invasion (CD31) and lymphatic invasion (D2-40). The H&E and IHC scores were collected by a single pathologist (EIH). Results were analyzed by kappa statistics, which correct for agreement by chance, and by percent agreement. Following analysis of data, the participating pathologists submitted and discussed individual criteria used in the diagnosis of LVI.

The statistical analyses of this study were focused on the estimation of the agreement rate and Kappa Statistics among six GI pathologists. The estimation of the agreement rate was based on the Binomial distribution. Kappa Statistics is a quality index, which compares the observed agreement with agreement expected by chance. Agreement expected by chance is determined by assuming each pathologist made their ratings randomly but with probabilities equal to the overall proportions or marginal frequencies. The possible values of Kappa range from +1 (perfect agreement) via 0 (no agreement above that expected by chance) to −1 (complete disagreement). The point estimates and the 95% confidence intervals of the agreement rate as well as Kappa statistics were reported.

RESULTS

The overall average observed agreement amongst all six pathologists using H&E slides and immunohistochemical stains was fair (agreement: 62.53; kappa (κ)= 0.24). One pathologist

had a noted tendency to diagnose positive lymphovascular invasion, while two pathologists were very conservative in the diagnosis.

The average kappa values were determined separately for the H&E slides (large and small vessel), CD31 (large and small vessel), and D2-40 (small vessel) (Figure 1). Interobserver agreement was fair for H&E small vessel invasion (κ= 0.28; 95%CI 0.22–0.34). The least agreement was seen in interpretation of H&E large vessel invasion (κ = 0.18; 95%CI 0.11–0.26). Agreement was not improved by use of immunohistochemical stains: CD31 (large vessel, κ = 0.42, 95%CI 0.20–0.63, small vessel, κ = 0.26, 95%CI 0.10–0.42) and D2-40 (κ = 0.32, 95%CI 0.21–0.42).

Values for observed agreement were similar to the kappa values above (Figure 2). However, observed agreement was highest using H&E stains and CD31 immunohistochemistry to evaluate large vessel invasion (H&E agreement = 76.80, 95%CI 65.24–88.36; CD31 agreement = 89.49, 95%CI 84.45–94.13). The least agreement was seen in evaluation of small vessel invasion on H&E stains (agreement = 66.40, 95%CI 62.95–69.85). Use of immunohistochemistry did not significantly improve agreement of small vessel invasion: CD31 (small vessel, agreement = 74.40, 95%CI 68.42–80.39) and D2-40 (agreement = 68.07, 95%CI 63.37–72.77).

DISCUSSION

Interobserver agreement in the diagnosis of LVI in Stage II colorectal carcinoma was poor on H&E examination and did not improve with addition of immunohistochemical markers of vascular and lymphatic endothelium. Notably, the interobserver agreement in evaluation of large vessel invasion on H&E was only slightly higher than would be seen by chance alone.

Several hypotheses exist to explain the discrepancies in diagnosis. A logical explanation would be that different criteria were applied by each pathologist. Following collection and analysis of data, the participating pathologists submitted and discussed the individual criteria for LVI. These criteria included: presence of tumor cells in a space lined by endothelial cells and/or containing erythrocytes; endothelial cells present either completely surrounding the tumor, or with only focal disruption; attachment of tumor cells to the vascular wall (Figure 3A); and large vessel invasion defined by presence of a muscular wall, either adjacent to tumor or disrupted by tumor. While individual criteria for small vessel invasion were concordant, the interpretation of large vessel invasion varied among pathologists. Three pathologists required only that tumor be present within or adjacent to the muscular wall (Figure 3B). The remaining three pathologists required the presence of an elastic lamina surrounding the tumor focus or a distinct disruption of the elastic lamina (Figure 3C).

This difference in evaluation of large vessel invasion does not fully explain the poor degree of interobserver agreement. Another explanation for variation includes interpretation and “threshold” for diagnosis. While one pathologist may be convinced that a small cluster of tumor in a space with a few red blood cells equals small vessel invasion, another might interpret the same findings as retraction artifact with carryover of erythrocytes. One pathologist may require only one or two criteria to determine LVI (i.e. a space with a few endothelial cells), while another requires the presence of most or all criteria. Another threshold for diagnosis includes the amount of LVI present on the slide. If only a single suspicious focus is present, the pathologist may be less likely to interpret the case as positive for LVI, as opposed to a case with extensive vessel invasion present. Alternatively, in a case with obvious tumoral retraction artifact, the pathologist may be less likely to interpret true LVI as positive.

An additional consideration is to the attentiveness of each pathologist while examining the slide. The participating pathologists undoubtedly spent a variable amount of time searching each slide for single cell or small cluster LVI.

Immunohistochemical stains for CD31 and D2-40 were used to aid in identifying small and large vessel invasion. CD31 is a marker of endothelial cells and is also designated “platelet endothelial cell adhesion molecule-1” (PECAM-1). D2-40 is a relatively new immunohistochemical stain that been demonstrated to label lymphatic endothelium but is unreactive with vascular endothelium (7,29).

Upon the addition of the IHC slides, all participating pathologists identified more cases with LVI than on initial H&E review. Interestingly, despite this increase, the interobserver agreement was unimproved. This may be partially attributable to the randomization of the slides; the de-identified slide numbers did not correspond between the H&E and IHC slides. Also, the original H&E slide was not available for review after examination of the corresponding IHC slide. It is possible that in practice, the addition of IHC would be helpful in the examination of an area suspicious for LVI.

IHC for CD31 was somewhat challenging to interpret without the accompanying H&E slide. CD31 will stain more than endothelial cells; reactivity will also be seen with macrophages, platelets, lymphocytes, neutrophils, and fibroblasts. CD31 also reacts with lymphatics. There was a moderate amount of background staining of stroma and inflammatory cells (Figure 4B). The condensed stroma around a tumor focus may have been interpreted as endothelium by some pathologists, and *vice versa.* Upon review by the participating pathologists, it was noted that vessels present within the invasive tumor itself were interpreted as vascular invasion by some participants (Figure 4C). The CD31 stain was confirmatory in several cases that were highly suspicious for LVI on H&E examination (Figure 4A).

D2-40 was found to be a relatively clean and interpretable stain. There was some background staining of stroma, especially myofibroblasts, and nerves that occasionally hindered interpretation (Figure 4E), and no observed staining of blood vessel endothelium. The IHC highlighted the abundant lymphatics present around the tumor, and served to identify single cell (Figure 4C) and small cluster lymphatic invasion by tumor. Interobserver variation in this case may be explained by threshold differences (one pathologist may be more willing to call a single cell in a lymphatic “positive for invasion”). An example of a controversial D2-40 immunohistochemical image is presented in Figure 4F. There are also basic differences in examination of the slide; invasion by a single cell is difficult to screen on low or medium power.

Formal interobserver studies of lymphovascular invasion in colorectal cancer have not been previously undertaken. An interobserver study of lymphatic invasion in breast cancer has been published with the conclusion that identification of lymphatic invasion is not a reliably reproducible prognostic finding (31). Studies of vascular invasion in colorectal cancer have found that the addition of an elastin stain may improve the diagnosis; however, formal interobserver agreement studies were not performed (32–34).

The results of this study highlight the necessity for improvement in our criteria for diagnosis of LVI and call into question all studies examining the association of LVI with patient outcome and prediction of response to treatment. The quest to improve interobserver agreement is a challenging one. The main barrier to improvement lies in the lack of a gold standard for LVI. The presence of metastases to lymph nodes and distant organs proves that LVI has occurred, but does not prove that a single focus on a single slide is necessary true LVI. The current recommendations from the 1999 CAP Consensus Statement include the presence of tumor in a vascular space lined by endothelium, attachment of tumor to the vascular wall, and presence of an elastic lamina surrounding a tumor focus (for large vessel invasion). Strict adherence to

these criteria, thus increasing the threshold for diagnosis of LVI, will improve agreement and decrease the reported positive cases for LVI. This will not necessarily improve patient care, as several studies have associated foci "suspicious for LVI" with increased risk for lymph node metastases. In the future, improved histochemical and immunohistochemical stains identifying blood vessels and lymphatics may also improve our diagnosis and agreement of LVI, and thus improve patient care.

Acknowledgements

This research was supported by a Specialized Program of Research Excellence (SPORE) in gastrointestinal cancer grant from the National Institutes of Health/National Cancer Institute (P50 CA95103).

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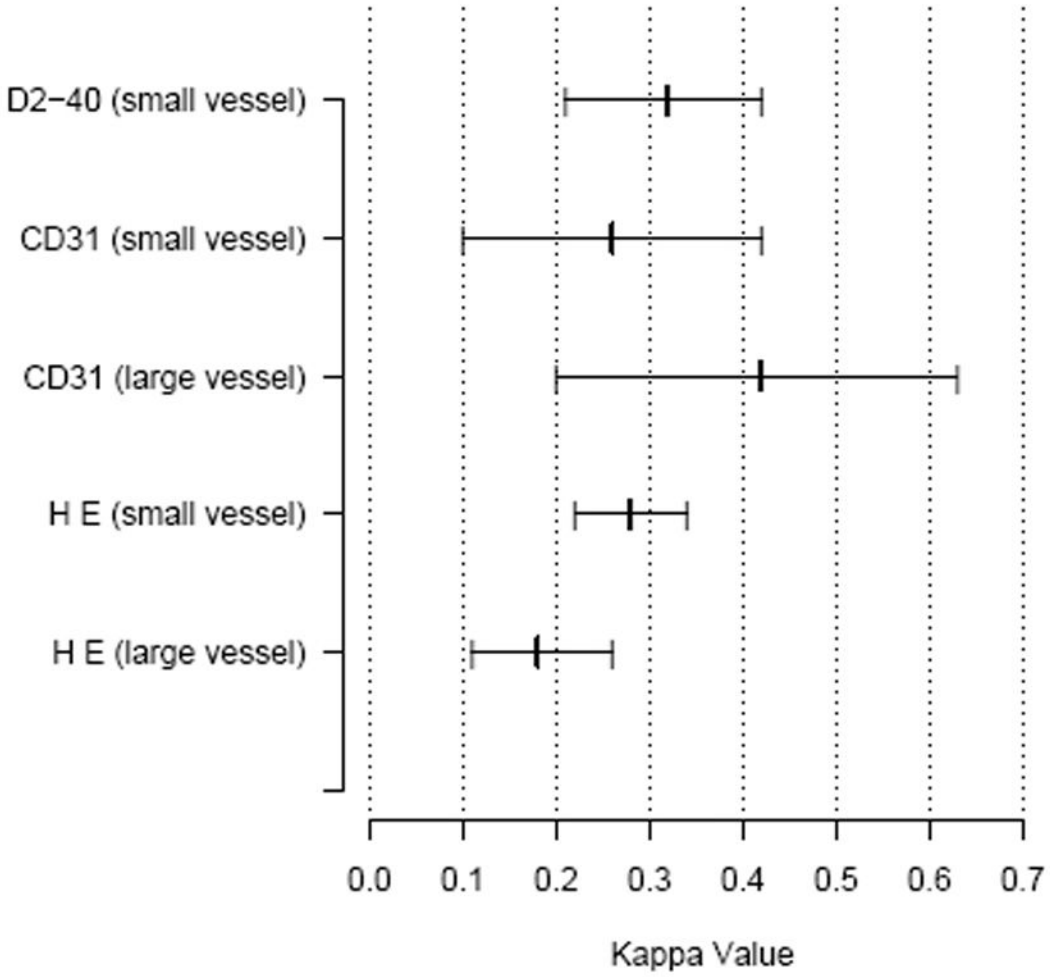

Figure 1. Interobserver kappa Values for Lymphovascular Invasion in Colorectal Cancer
[kappa graph with labels_Jan08]
Interobserver average kappa values show no significant improvement in agreement upon addition of immunohistochemical stains.

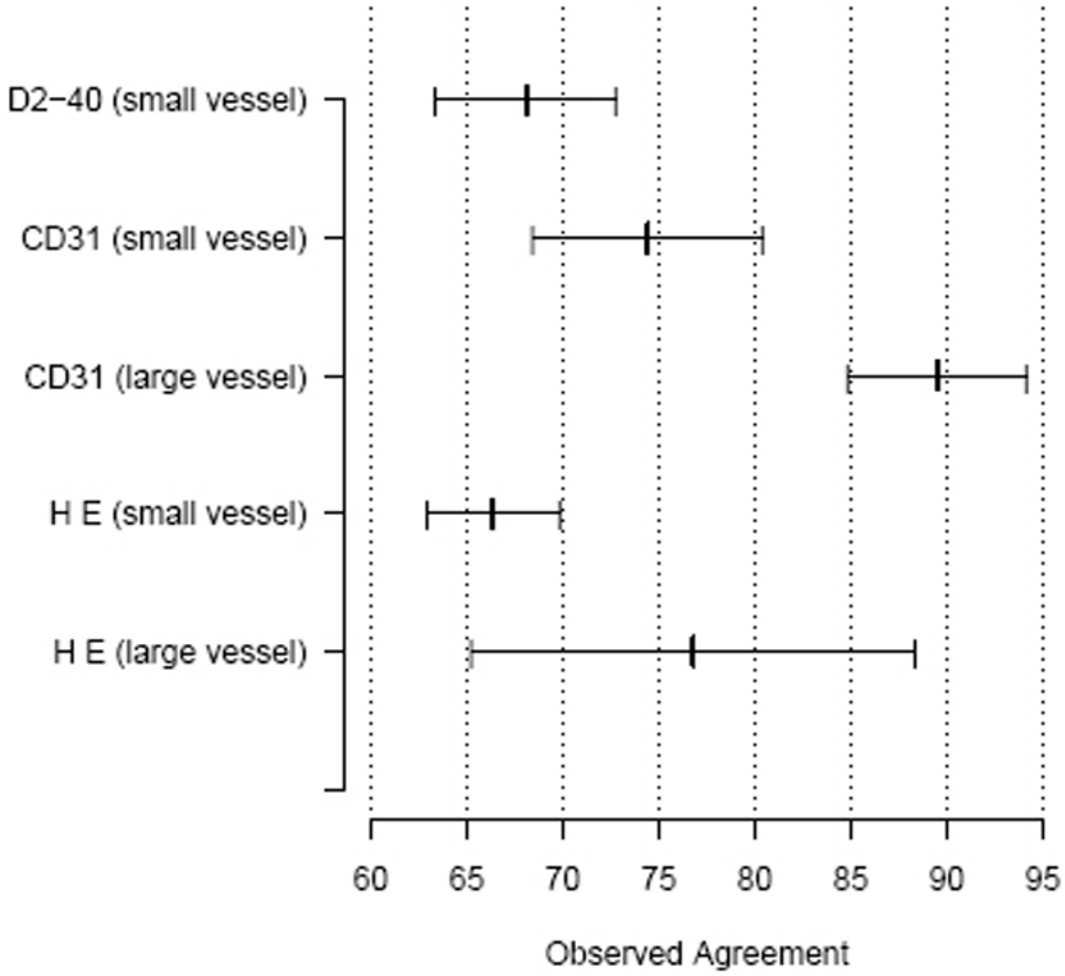

Figure 2. Interobserver Agreement Values for Lymphovascular Invasion in Colorectal Cancer
[agreement graph with labels_Jan08]
Observed agreement values show the highest agreement using CD31 immunohistochemistry to evaluate large vessel invasion.

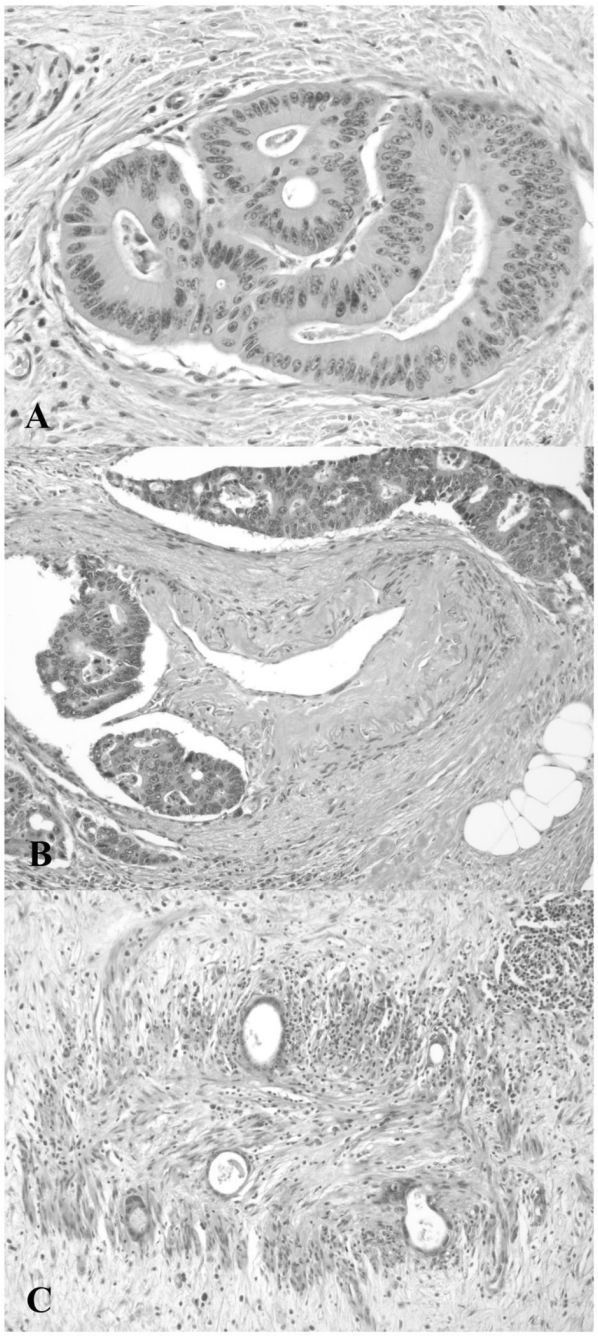

Figure 3.
A: An example of positive small vessel invasion, diagnosed by all six participating pathologists on H&E examination. H&E, 200×. B: Large vessel invasion by tumor. The tumor invades and disrupts the muscular wall of the vessel. H&E, 100×. C: Large vessel invasion: the tumor disrupts the muscular wall and elastic lamina of the obliterated blood vessel. H&E, 100×.

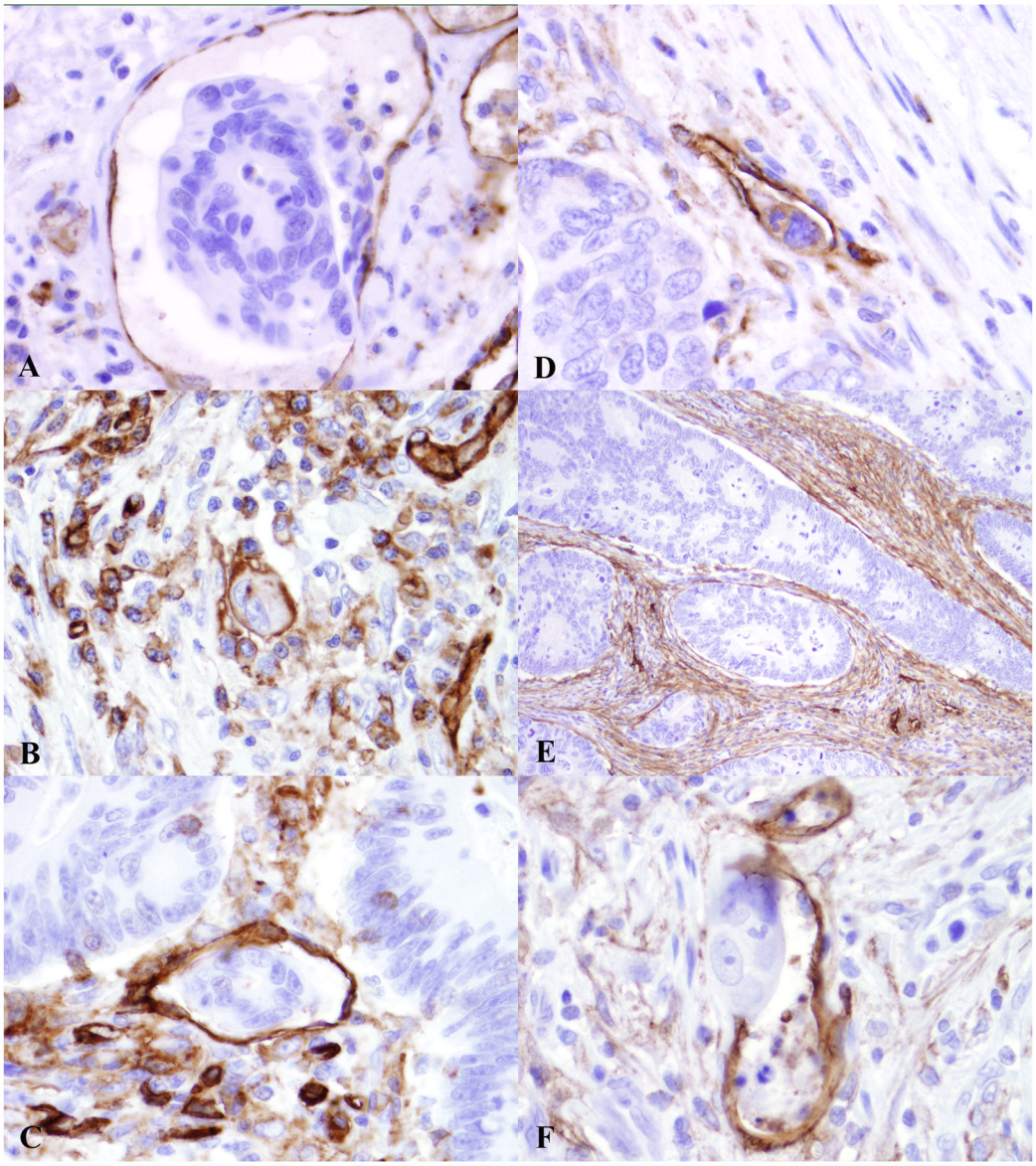

Figure 4.
A: An example of consensus agreement for small vessel invasion by tumor, as seen on CD31 immunohistochemical stain, 400×. B: Inflammatory cell staining in the background stroma may hinder a diagnosis of single cell small vessel invasion. CD31 immunohistochemical stain, 400×. C: This cluster of tumor cells was called positive for small vessel invasion by some participating pathologists due to the strong immunohistochemical staining for CD31; others considered the case to be negative because the cluster was within the bulk of the tumor mass. CD31 immunohistochemical stain, 400×. D: Single-cell lymphatic invasion by tumor highlighted on D2-40 immunohistochemistry, 400×. E: Background stromal fibroblast staining by D2-40 immunohistochemistry, 100×. F: A cluster of tumor cells is identified within a space showing incomplete D2-40 immunoreactivity; the majority of participating pathologists considered this to be positive lymphatic invasion. D2-40 immunohistochemistry, 400×.